Original Article

Development of a Novel PmpD-N ELISA for *Chlamydia psittaci* Infection*

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Abstract

Objective *Chlamydia psittaci* is an avian respiratory pathogen and zoonotic agent. The wide prevalence of *C. psittaci* poses a threat to the poultry industry and its employees. However, few commercial kits are available for detecting avian antibodies excluding the in-house ELISA kit. In this study, we developed a novel ELISA kit for detecting antibodies against *C. psittaci* based on the N-terminal fragment of polymorphic outer membrane protein D (PmpD-N) as the coating antigen.

Methods The antigen concentrations, primary antibody, and cut-off value were determined and optimized. The ELISA, designated PmpD-N ELISA, was assessed for sensitivity, specificity, and concordance using sera samples from 48 experimentally infected and 168 uninfected SPF chickens.

Results The sensitivity and specificity of PmpD-N ELISA were 97.9%, 100%, respectively, while the concordance was 98.1% as compared to that of MOMP-ELISA. No cross-reaction with positive sera for other avian pathogens was found. Using PmpD-N ELISA, 799/836 clinical samples were positive, including 93.0% and 98.1% positivity in layers and broilers, respectively.

Conclusion These data indicate that indirect ELISA with PmpD-N as the antigen candidate is a promising approach for the surveillance of *C. psittaci* infection.

Key words: *Chlamydia psittaci*; Indirect ELISA; N-terminal fragment of polymorphic membrane protein D; Seroprevalence

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INTRODUCTION

The obligate intracellular gram-negative bacterium *Chlamydia psittaci* (*C. psittaci*) causes systemic disease in psittacine birds, domestic poultry, and wild fowl[1]. In poultry, the pathogen often induces a disease called ornithosis, generally referred to as chlamydiosis in other bird species. Importantly, *C. psittaci* is also a zoonotic pathogen that can cause ocular adnexal lymphomas[2], pneumonia, encephalitis, endocarditis, and even death in humans[3]. Thus, chlamydiosis is associated with severe economic losses in the poultry industry, and it also poses a serious health

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hazard to humans who are in close contact with infected birds[4-5]. *C. psittaci* strains in poultry have been reported in many countries, including Austria[6], France[7], China[8], and Germany[9]. Moreover, a high seroprevalence was recorded in Belgian and Northern French chicken farms using an indirect ELISA, with seropositivity rates of 96% and 90% in broiler and layer flocks, respectively[10]. In a recent study, *C. psittaci*-specific serum antibodies and *C. psittaci* antigens were detected in 10.0% and 26.7% of birds, respectively, suggesting that *C. psittaci* prevalence in Beijing is similar to that in European cities[11].

The complement fixation test (CFT) is currently used in routine diagnosis[12]. An indirect hemagglutination assay (IHA) is commercially available for detecting *Chlamydia* antibodies in China[13]. Unfortunately, the IHA lacks species specificity and sensitivity because of the use of inactivated whole *Chlamydia* elementary bodies as the diagnostic antigens. Additionally, both CFT and IHA require experienced technicians to interpret the results. The true seroprevalence of *C. psittaci* and potential risks are underestimated. An in-house ELISA kit based on recombinant MOMP has been developed and evaluated, with 100% sensitivity and specificity for chicken *C. psittaci* survey[14]. In addition to major outer membrane protein (MOMP), polymorphic membrane proteins (Pmps) are classified as autotransporters and are highly immunogenic[15]. Among the Pmps, the N-terminal fragment of PmpD (PmpD-N) is an attractive candidate because it is an immunogenic and a conserved protein among *C. psittaci* strains. More importantly, PmpD expression can be detected within 24 h postinfection[16]. Therefore, a reliable, sensitive, and specific test is urgently required for the preliminary survey of avian *C. psittaci.*

In the present study, we evaluated the sensitivity and specificity of PmpD-N ELISA for detecting avian *C. psittaci* antibodies. Moreover, 836 bird blood samples from 14 intensive-raising farms in seven provinces were collected to determine the seroprevalence using PmpD-N ELISA.

MATERIAL AND METHODS

Cell Culture and C. psittaci Preparation

Buffalo Green Monkey Kidney (BGMK) cells used for the propagation of *C. psittaci* stocks were donated by Professor WANG Cheng Ming, Yangzhou University, China. The mild-virulence *C. psittaci* strain CB7 (genotype A) originally isolated from a wild bird in Wuhan, China[17] was purchased from the China Institute of Veterinary Drug Control (IVDC, Beijing, China), inoculated into BGMK monolayers, and titrated according to standard protocols[18]. These isolates were inoculated into BGMK monolayers and titrated according to standard protocols. The standardized aliquots were frozen at -80 °C until use.

Serum Samples

A total of 168 SPF chickens (Vital Merial Experimental Animal Co., Ltd, Beijing, China) were selected for the immunization and comparative tests. Negativity for *C. psittaci*-specific antibodies was confirmed by both the in-house ELISA kit[14] and IHA kit (Lanzhou Veterinary Research Institute, Chinese Academy of Agriculture Science). Twenty-four SPF chickens were kept in an isolated facility and infected intra-tracheally with 0.1 mL of $5 \times 10^{8.5}$ IFUs of the CB7 strain[17]. Sera from the chickens were sampled on days 0, 3, 7, and 13 post-infection.

A total of 836 chicken sera were collected randomly from 14 chicken farms in seven provinces across China, including 415 sera from laying hens aged 130-500 days and 421 sera from broiler breeder flocks aged 100-450 days. In China, 90% of all farm-raised chickens are raised in an all-in all-out production system. To ensure representative sampling, the 14 chicken farms were selected among 150 intensive poultry cooperatives listed by the Ministry of Agriculture of China using a two-stage cluster procedure. Blood samples were stored for 1 h at 37 °C. Sera were collected after centrifugation (325 ×*g*, 10 min, 4 °C) and stored at -20 °C before analysis.

Gene Cloning, Expression, and Purification of PmpD-N

Primers specific for *pmpD*-N were designed using Oligo 7 (Molecular Biology Insights, USA) based on the 6BC strain. The forward primer (PmpDF) sequence was 5'-CCGGAATTCATGGGATCCAATGT GTTGATTTCTGGAA-3' (*Eco*RI site is underlined), and the reverse primer (PmpDR) sequence was 5'-CCGCTCGAGTCAAACAGCCCCACCTGTAGGAGCA-3' (*Xho*I site is underlined). The genomic DNA of the CB7 strain was extracted using a QIAamp® DNA Mini Kit (Qiagen Ltd., Crawley, UK), according to the manufacturer's instructions. *pmpD*-N was amplified

from DNA by PCR using PmpDF and PmpDR. PCR amplification of *pmpD*-N was performed in a thermal cycler with an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR product was cloned into the *Eco*RI and *Xho*I sites of pET30a (Novagen, Madison, WI, USA). The size and orientation were confirmed by restriction endonuclease digestion analysis and DNA sequencing of purified plasmid DNA. Then, the plasmid was transformed into *Escherichia coli* BL21 (DE3) (Invitrogen, CA, USA), and recombinant bacteria were selected on LB broth agar containing 50 μg/mL kanamycin. For large-scale PmpD-N production, the positive clone was grown overnight at 37 °C in 5 mL of LB supplemented with 50 μg/mL kanamycin. After overnight shaking at 37 °C, 2 mL of bacteria was added to 200 mL prewarmed LB with 50 μg/mL kanamycin. After 2 h of shaking at 37 °C, IPTG was added at a final concentration of 1 mmol/L. Following an additional incubation for 5 h at 30 °C, the bacterial cells were harvested by centrifugation. The cells were re-suspended in PBS at 2 mL/g wet weight. Lysozyme (1 mg) and benzonase nuclease (3 U/mL) were added. After incubation on ice for 30 min, the cellular debris was pelleted by centrifugation at 5000 ×*g* for 30 min at 4 °C. The supernatant was passed through a Ni-NTA column equilibrated with binding buffer (PBS containing 10 mmol/L imidazole). The column was then washed with washing buffer (PBS containing 20 mmol/L imidazole). The expressed PmpD-N protein was eluted three times using elution buffer (PBS containing 250 mmol/L imidazole). The purified protein was quantified using the NANODROP 2000 Spectrophotometer (Gene Ltd., USA).

Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis and Western Blotting Assay

Protein extracts from BL21 cells were mixed with an equal volume of reduced Laemmli sample buffer, boiled for 10 min, and separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated protein was either stained by standard methods using Coomassie brilliant blue or transferred onto polyvinylidene difluoride membranes. The proteins were then reacted with a 1:1000 dilution of the *C. psittaci* strain 6BC-specific polyclonal antibodies (unpublished) and a 1:4000 dilution of horseradish peroxidase (HRP)-labeled goat-anti chicken IgG (Sigma-Aldrich, Shanghai, China) for 1 h at room temperature. The membrane was developed with the chromogenic substrate 3,3′-diaminobenzidine containing 0.03% hydrogen peroxide (eBioscience, Laizee Biotech Co., Ltd, China).

Optimization of PmpD-N ELISA

The optimal concentration of the coating antigen PmpD-N and the optimal dilution of test sera were determined via checkerboard titration on a 96-well ELISA plate. Antigens were diluted from 1 to 10 μg/mL, whereas 6BC-specific polyclonal antibodies and negative sera from SPF chickens were prepared at dilutions of 1:25 to 1:800. The highest dilution of antigen was considered optimal when the optical density ($OD_{450/630}$) was approximately 1.0 and the dilution provided maximum contrast between the aforementioned positive and negative sera[19-20].

The indirect ELISA was performed using the following procedure[21-22]. First, 96-well ELISA plates were coated with 100 μL/well of the purified PmpD-N protein diluted in 0.05 mol/L bicarbonate/carbonate buffer (pH 9.6) at 4 °C overnight. Subsequently, the plates were washed four times with PBS containing 0.05% Tween 20 to remove the unbound antigen, and then the wells were blocked at 37 °C for 2 h using 200 μL of blocking buffer (PBS containing 5% skimmed milk), followed by an additional washing step. Then, 100 μL of sera diluted in PBS were added into each well and incubated for 1 h at 37 °C. HRP-conjugated goat anti-chicken (HRP-IgG) antibodies (Sigma-Aldrich, Shanghai, China) were diluted 1:10,000 in PBS, and 100 μL was added to each well. After incubation and washing steps were performed as described previously, the colorimetric reaction was started by the addition of 100 μL/well 3,3,5,5′-tetramethylbenzidine (Qiagen, Stuttgart, Germany) at 37 °C for 10 min, and 2 mol/L H_2SO_4 was used to stop color development. The plates were read using a universal Microplate Reader (Thermo Life Sciences, Shanghai, China) at 450 nm/630 nm. The cut-off value was calculated from the result of 144 sera from SPF chickens proved negative by both IHA and MOMP ELISA using the following formula mean OD + [3 × standard deviation (3SD)]. A serum sample was considered positive when its OD exceeded the cut-off value.

Evaluation of Sensitivity, Specificity, and Concordance

To evaluate the sensitivity, specificity, and concordance, serum samples infected with the CB7

strain for 7 and 13 days and 168 SPF chicken sera described previously were tested using PmpD-N ELISA and in-house MOMP ELISA kits (Dr. Daisy Vanrompay, Department of Molecular Biotechnology, Faculty of Bioscience Engineering, Ghent University). The sensitivity, specificity, and concordance were calculated using the following formulae: sensitivity = [number of true positives/(number of true positives + number of false negatives)] × 100%; specificity = [number of true negatives/(number of true negatives + number of false positives)] × 100%; concordance = (the sum of positive-positive values and negative-negative values)/the total number of serum samples × 100%.

To determine whether this test has cross-reaction with other chlamydiae antibodies, PmpD-N proteins of other different chlamydiae, including *Chlamydia abortus*, *Chlamydia felis*, *Chlamydia caviae*, *Chlamydia avium*, *Chlamydia gallinacean*, *Chlamydia pecorum*, *Chlamydia muridarum*, *Chlamydia trachomatis*, *and Chlamydia suis*, were used to BLAST with that of *C. psittaci.*

Sera positive for *infectious bursal disease virus* (*IBDV*), *herpesvirus of turkeys* (*HVT*), *H9N2 influenza virus* (*H9N2*), *avian metapneumovirus* (*aMPV*), *avian leukosis virus* (*ALV*), *Ornithobacterium rhinotracheale* (*ORT*), and *Haemophilus paragallinarum* (*Hp*) were purchased from IVDC. The specific test with the aforementioned eight positive sera was further used to determine the specificity of the PmpD-N ELISA assay.

Early Infection Establishment

Chicken serum samples infected with the CB7 strain for 3 and 7 days were tested using both PmpD-N ELISA and in-house MOMP ELISA kits to test whether PmpD-N ELISA could detect early infection by *C. psittaci*.

RESULTS

Cloning, Expression, and Identification of PmpD-N

The amplified PCR products of the gene that encodes PmpD-N were sequenced, and the expected size of 1021 bp was observed. The PCR products were cloned into the PET-30a vector. The recombinant plasmids of the correct size were identified by PCR and gene sequencing. The pET30-PmpD-N plasmid was overexpressed successfully in *E. coli* host cells. Protein bands were subsequently visualized by Coomassie brilliant blue staining (Bio-Rad, Life Science, Beijing, China). SDS-PAGE revealed the PmpD-N fusion protein with an approximate molecular mass of 45 kD (including a 5.4 kD His tag) (Figure 1A), which was consistent with the expected size of the PmpD-N fusion protein. The expressed protein was then analyzed by an immunoblotting assay using 6BC polyclonal antibodies. *C. psittaci*-specific sera reacted with the PmpD-N fusion protein, and an approximate molecular mass of 45 kD was observed (Figure 1B). No *Chlamydia*-specific proteins were detected in lysates derived from pET30a-transformed *E. coli* cells.

Optimization of PmpD-N ELISA

Checkerboard titration was used to determine the optimal dilutions of antigens and antibodies. The optimal PmpD-N protein concentration was 4 μg/mL (Figure 2) for the coating antigen, and the dilution of the tested sera was determined to be 1:50 (Figure 2). Under these conditions, the *P*/*N* value was maximal (49.3).

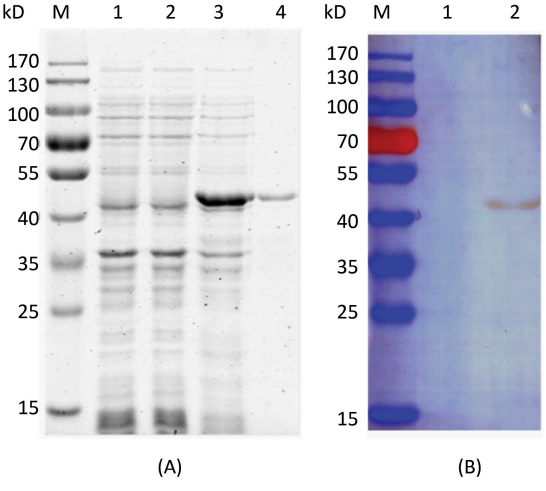

Figure 1. Identification of the PmpD-N protein from the pET30-PmpD-N plasmid by SDS-PAGE. (A) M, molecular weight marker; 1, *Escherichia coli* expressing the pET30a vector; 2, *E. coli* expressing pET30-PmpD-N before induction; 3, *E. coli* expressing pET30-PmpD-N at 3 h post-induction; and 4, purified pET30-PmpD-N protein. Detection of recombinant PmpD-N fusion protein by Western blotting with polyclonal antibodies against *C. psittaci* 6BC. (B) M, molecular weight marker; 1, *E. coli* expressing the pET30a vector; and 2, *E. coli* expressing pET30-PmpD-N protein.

Determination of the Cut-off Value

The average OD of the 144 negative sera was 0.0306, whereas the SD was 0.0048. Therefore, the cut-off OD value was 0.045 (mean + 3SD). Thus, OD values equal to or exceeding the cut-off value were confirmed to be positive, whereas those less than 0.045 were considered negative in this assay.

Sensitivity and Specificity

Serum analysis via PmpD-N ELISA revealed that 47 of 48 *Chlamydia*-infected chicken sera were positive, whereas one sample was negative, resulting in a sensitivity of 97.9%. By contrast, 45 of the 48 samples (93.8%) were positive using the MOMP ELISA kit. In addition, all 168 of the negative SPF samples were confirmed to be negative by both methods, yielding a specificity of 100% (Table 1).

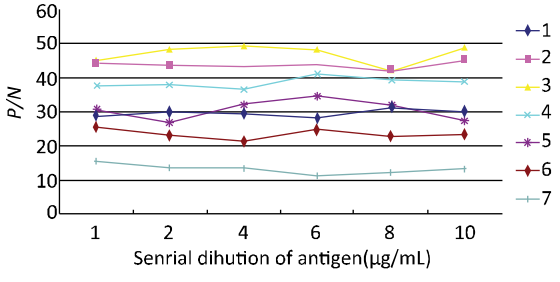

Figure 2. Optimization of the antigen and serum at different dilutions. Line 1, 1:12.5 dilution; Line 2, 1:25 dilution; Line 3, 1:50 dilution; Line 4, 1:100 dilution; Line 5, 1:200 dilution; Line 6, 1:400 dilution; and Line 7, 1:800 dilution.

Assessment of Cross-reactivity

Amino acid sequence alignment of different chlamydiae illustrated that the similarity between *C. psittaci* and other different chlamydiae ranged 34.3%-87% (Figure 3).

All OD values were below the defined cut-off point (Table 2). No cross-reactivity was found in tests with positive sera against *IBDV*, *HVT*, *H9N2*, *aMPV*, *ALV*, *ORT*, and *Hp.*

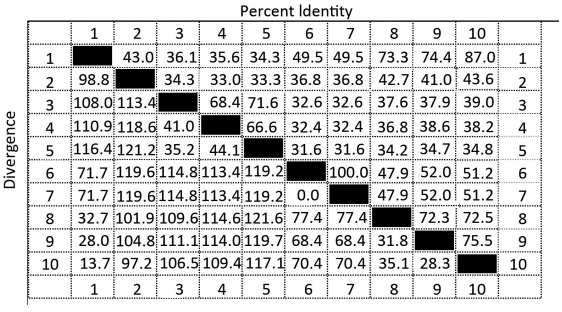

Figure 3. Amino acid sequence alignment among different chlamydiae. No. 1, *Chlamydia psittaci*; No. 2, *Chlamydia pecorum*; No. 3, *Chlamydia muridarum*; No. 4, *Chlamydia trachomatis*; No. 5, *Chlamydia suis*; No. 6, *Chlamydia avium*; No. 7, *Chlamydia gallinacean*; No. 8, *Chlamydia felis*; No. 9, *Chlamydia caviae*; and No. 10, *Chlamydia abortus*.

Table 1. Sensitivities and Specificities of Serological Detection of *Chlamydia psittaci* Antibodies in Chickens

Assay	True Positives[a] (*n*=48)		True Negatives[b] (*n*=168)		Sensitivity[c] (%)	Specificity[d] (%)
	Positive	Negative	Positive	Negative		
PmpD-N ELISA	47	1	0	168	97.9	100
MOMP-ELISA	45	3	0	168	93.8	100

Note. [a]Sera obtained from *C. psittaci*-infected chickens were tested using the indirect ELISA kit on days 7 and 14 post-infection. [b]Sera collected from SPF chickens were assessed using the IHA and in-house indirect ELISA kits. [c]Sensitivity = [number of true positives/(number of true positives + number of false negatives)] × 100%. [d]Specificity = [number of true negatives/(number of true negatives + number of false positives)] × 100%.

Table 2. Specificity Test Using Positive Sera of Other Avian Pathogens

Positive Sera	*IBDV*	*HVT*	*H9N2*	*aMPV*	*ALV*	*REV*	*ORT*	*Hp*
OD values	0.022	0.023	0.039	0.033	0.032	0.02	0.027	0.038

Note. Sera positive for *infectious bursal disease virus* (*IBDV*), *herpesvirus of turkeys* (*HVT*), *avian influenza virus serotype H9N2* (*H9N2*), *avian metapneumovirus* (*aMPV*), *avian leukosis virus* (*ALV*), *Ornithobacterium rhinotracheale* (*ORT*), and *Haemophilus paragallinarum* (*Hp*) were used to determine the specificity of PmpD-N ELISA.

Concordance between PmpD-N ELISA and the MOMP ELISA kit

In total, 47 of the 48 positive sera identified by PmpD-N ELISA were confirmed to be positive, whereas three samples were found to be negative using the MOMP ELISA kit. On the contrary, all 168 negative sera were confirmed using the MOMP ELISA kit (Table 3). Hence, the concordance was 98.1% between the two assays.

Establishment of Early Infection

Among the 24 chicken serum samples on day 3 post-infection, 10 sera identified by PmpD-N ELISA were confirmed to be positive, whereas none was found to be positive using MOMP ELISA (Table 4). All 24 chicken serum samples on day 7 post-infection were confirmed to be positive by PmpD-N ELISA, and three sera remained negative according to MOMP ELISA (Table 4).

Table 3. Concordance between PmpD-N ELISA and MOMP ELISA for Experimentally Infected Chickens

PmpD-N ELISA	MOMP ELISA	
	Positive	Negative
Positive	44	3
Negative	1	168
Concordance[a]	98.1%	

Note. [a]Concordance = (the sum of positive-positive values and negative-negative values)/the total number of serum samples × 100%.

Clinical application of PmpD-N ELISA

A total of 836 chicken serum samples from seven different provinces were tested for *C. psittaci* antibodies using the PmpD-N ELISA and in-house MOMP ELISA kits (summarized in Table 5). The results of the two methods were consistent. In total, 799 of the 836 sera (95.6%) were determined to be positive, whereas 37 samples were found to be negative using PmpD-N ELISA. With respect to the regions, high seroprevalence was found in Northern China with the intensive raising-farm system, whereas negative samples were found in Henan, Central China. All 98 samples (100%) from Heilongjiang province, 28 samples (100%) from Liaoning province, and 212 samples (100%) from Shandong province, as well as 157 of the 159 samples (98.7%) from Tianjin, 230 of the 232 samples (99.1%) from Hebei province, and 74 of the 80 samples (92.5%) from Beijing were found to be seropositive in the study.

Table 4. Serological Detection of *C. psittaci* Antibodies in Chickens on Days 3 and 7 Post-infection

Assay	Sera[a] On day 3		Sera[b] On day 7	
	Positive	Negative	Positive	Negative
PmpD-N ELISA	10	14	24	0
MOMP ELISA	0	24	21	3

Note. [a]Sera obtained from *C. psittaci*-infected chickens were tested using the indirect ELISA kit on day 3 post-infection. [b]Sera obtained from *C. psittaci*-infected chickens were tested using the indirect ELISA kit on day 7 post-infection.

Table 5. Serological Detection of Clinical Serum Samples Using PmpD-N ELISA and MOMP ELISA

Province	Species	No.[A]	PmpD-N ELISA			MOMP ELISA		
			Positives	Negatives	Positive rate (%)	Positives	Negatives	Positive rate (%)
Heilongjiang	Breeding broilers	98	98	0	100.0	95	3	96.9
Liaoning	Breeding broilers	28	28	0	100.0	26	2	92.9
Tianjin	Breeding layers	159	157	2	98.7	148	11	93.1
Hebei	Breeding broilers	232	230	2	99.1	227	5	97.8
Beijing	Layers	49	49	0	100.0	49	0	100.0
	Breeding broilers	31	25	6	80.6	24	7	77.4
	Layers	180	180	0	100.0	175	5	97.2
Shandong	Breeding broilers	32	32	0	100.0	30	2	93.8
Henan	Layers	27	0	27	0.0	0	27	0.0
Total	Layers	415	386	29	93.0	372	43	89.6
	Breeding broilers	421	413	8	98.1	402	19	95.5

Note. [A]Total of 495 blood samples were randomly collected from 4 intensive pig farms.

Among the chickens sampled, 386 of the 415 layers (93.0%) were found to be positive, and 413 of the 421 broiler sera (98.1%) were positive. No significant difference was observed between laying hens and breeding broilers.

DISCUSSION

The aim of this study was to develop an affordable and reliable kit for the detection of avian *C. psittaci* infection, as well as to develop a method to analyze the immune status of vaccinated flocks. In this study, PmpD-N ELISA was optimized as an indirect ELISA assay, which was demonstrated to be highly sensitive (97.9%) and specific (100%) for detecting *C. psittaci* antibodies. The concordance was 98.1% compared with the MOMP ELISA kit. Further studies demonstrated that no cross-reaction was found for sera positive for eight other chlamydiae. Therefore, PmpD-N ELISA is a promising approach for the serological diagnosis of avian *C. psittaci*. Its sensitivity and specificity were comparable with those of the in-house MOMP ELISA kit.

The merit of PmpD-N as the coating antigen is that it is a highly conserved fragment in the polymorphic outer protein membranes of *C. psittaci*, which makes it comparable with *C. psittaci*-specific MOMP as a diagnostic candidate. As it is difficult for us to obtain highly pure antibodies against other chlamydiae, PmpD-N proteins of other different chlamydiae were used to blast with that of *C. psittaci* to assess cross-reaction. The similarity of the amino acid sequence between *C. psittaci* and other chlamydiae ranged 34.3%-87%, suggesting that the established PmpD-N ELISA might be specific for detecting *C. psittaci* antibodies. In comparison with *C. trachomatis* and *C. pneumonia*, PmpD-N of *C. psittaci* translocates to the surface of the bacterium, where it noncovalently binds to other components of the outer membrane. Antibodies against PmpD-N were neutralizing. Recombinant PmpD-N also stimulated monocyte activation[23]. A recombinant HVT vaccine expressing PmpD-N of *C. psittaci* strain CB7 (rHVT-*pmpD*-N) was confirmed to express PmpD-N by immunoblotting and immunofluorescence, and it may be viable as a candidate dual vaccine that provides protection against both extremely virulent MDV and *C. psittaci*[24]. Clustering analysis resolved PmpD proteins into three distinct clades with *C. abortus* Pmp18D, being most similar to those originating from *C. psittaci*, *C. felis*, and *C. caviae*[15]. More interestingly, PmpD ELISA might be applied to detect early *C. psittaci* infection because of PmpD expression within 24 h of infection[16]. In the current study, *C. psittaci*-specific antibodies were detected on day 3 post-infection, whereas antibody positivity was not confirmed until day 7 post-infection using the in-house MOMP ELISA kit. Furthermore, PmpD-N ELISA is superior to the IHA kit, and the later kit usually gives rise to high false-positive test results because of antigenic cross-reactivity among *C. abortus*, *C. psittaci*, and *C. pecorum*, as well as with some gram-negative bacteria. In a recent report, 12.4% of adult pet birds and 4.85% of juvenile pet birds were found to be seropositive for *C. psittaci* using an IHA approach[13]. In this sense, IHA is not suitable for detecting *C. psittaci* prevalence because of the lack of differentiation between *C. psittaci* and other chlamydia species in poultry in addition to poor sensitivity.

C. psittaci infection is often neglected and misdiagnosed because of the lack of a commercially available *C. psittaci*-specific test kit. Infection transmission from raised turkeys and ducks to humans has been previously reported[7]. Sera from Belgian and French chicken farms were also proven to be *C. psittaci*-positive via an indirect ELISA based on major outer membrane protein, and the positive rate was extremely high (96% in broilers and 90% in layers)[10]. The positive rate coincided with our report, which yielded 93.0% positivity in layers and 98.1% positivity in broilers. Three main factors contribute to the high seroprevalence. First, no quarantine measures are implemented for breeding boilers and breeding layers imported to China from European and other countries. A large population of breeding broilers relies on supply from international enterprises every year, and high seropositivity has been confirmed in European poultry stocks. Second, direct contacts with infected birds may be another important cause due to the lack of biosecurity measures. The infected birds could shed *C. psittaci* in their feces, respiratory secretions, saliva, and feather dust, and healthy birds could then be infected by this contamination[1]. Third, live vaccines are contaminated with *C. psittaci*, thus contributing to the transmission of *C. psittaci* among avian species, as *C. psittaci* was found in SPF eggs with the positive rates of 33.3% and 31.7%[25].

In conclusion, PmpD-N ELISA coated with the PmpD-N protein was successfully developed, and this technique is a good candidate method for *C. psittaci* diagnosis. More importantly, PmpD-N ELISA

is comparable with MOMP ELISA with respect to sensitivity and specificity as well as concordance. It can be applied to identify *C. psittaci* infection.

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